Molluscicidal and antibacterial compounds from *Petunia hybrida*†

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Abstract: Three novel and six known petuniasterones were isolated from the aerial parts of *Petunia hybrida* (Solanaceae). In addition, chromatography yielded four groups of novel 2,3,4,6-tetra-*O*-acylated sucrose ester isomers. The sucrose esters showed molluscicidal and bactericidal activities. Petuniasterones had different molluscicidal activities, depending on the side-chains.

Keywords: *Petunia hybrida;* petuniasterones; 2,3,4,6-tetra-*O*-acylated sucrose esters; molluscicidal activity; antibacterial activity

1 INTRODUCTION

Schistosomiasis (bilharziasis) is one of the most frequent tropical parasitic diseases. Approximately 200 million people suffer from this disease, while 600 million run the risk of becoming infected. Schistosomiasis is caused by different species of the trematode *Schistosoma*, which live in the abdominal blood vessels of infected persons. The schistosomes lay eggs which are excreted with faeces or urine and reach water where miracidia larvae hatch out of the eggs and infest freshwater snails. Once in these intermediate hosts, the miracidia divide and produce thousands of cercaria which are shed into the water. These can then penetrate the skin of humans in contact with the contaminated water and, on reaching the blood vessels, develop into adult schistosomes.

Although chemotherapy is a valuable method of controlling the disease, the risk of re-infection remains, and there is still a need for selective and efficient molluscicides to eliminate the snail hosts and interrupt the parasite's life cycle. In our search for naturally occurring pesticides, *P hybrida* extracts have been found to contain molluscicidal compounds. In addition, some of the compounds exhibited antibacterial properties.

2 MATERIALS AND METHODS

2.1 Analysis

2.1.1 *NMR analysis*

NMR spectra were recorded on a Varian ^{Unity}Inova spectrometer (500 MHz).

2.1.2 *MS analysis*

Mass spectra were recorded on a Finnigan MAT TSQ 700 spectrometer.

2.1.3 *Chromatography*

The MPLC system consisted of a Büchi Chromatography pump, Waters Differential Refractometer R401, Pharmacia Single Path Monitor UV-1 (254 nm), LKB Radi Rac Fraction Collector, Latek R60 SV injection valve, KPG glass column (400 × 45 mm) with 300 g Merck silica gel 60 (43–60 μm), and a glass column (200 × 45 mm) with 125 g Merck RP-18 (25–40 μm). The HPLC system consisted of a Knauer HPLC PUMP 64, ABI Applied Biosystems 785A Programmable Absorbance Detector, Philips PM 8222 Dual-Pen Recorder, Bischoff HPLC column (250 × 4 mm) with Merck LiChrospher 100 RP-18 (5 μm) and Rheodyne 7125 injection valve with 20 μl loop for analytical HPLC; a Bischoff HPLC column (250 × 20 mm) with Merck LiChrospher 100 RP-18 (5 μm), Merck LiChroCart 250-10 HPLC cartridge with LiChrospher 100 RP-18 (10 μm) and Rheodyne 7125 injection valve with 500-μl loop for semi-preparative HPLC.

Merck silica gel 60 F254 aluminium sheets (0.2 mm) were used for TLC.

2.2 Plant extracts

2.2.1 *Plant material*

Dried and ground aerial parts of *Petunia hybrida* Vilm var *grandiflora* cultivated in Kenya were received in 1992 from Dr TO Midiwo, University of Nairobi. A voucher specimen has been deposited in the herbarium of the University of Nairobi, Kenya

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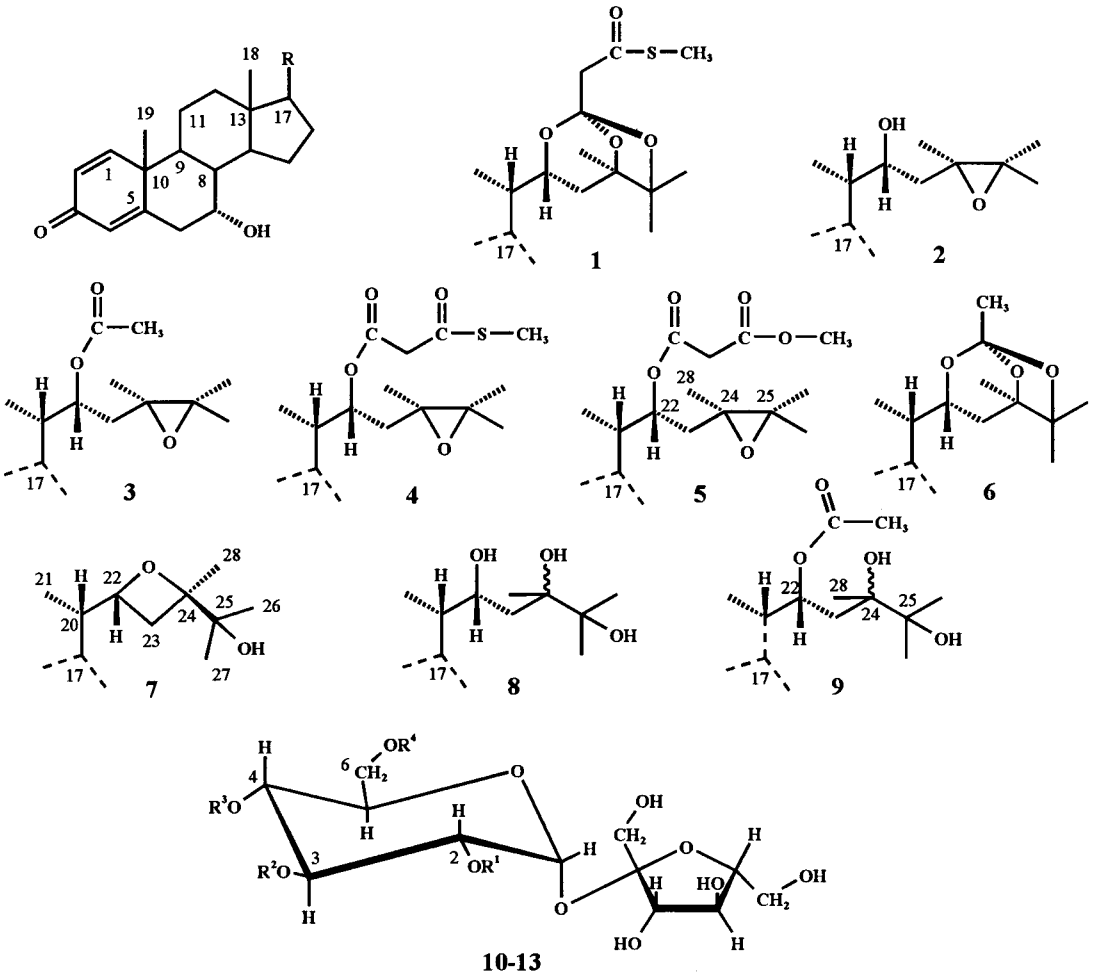
(voucher number: Nairobi University Herbarium (International Code Number NAI), Mathenge/ Midiwo 91/35).

2.2.2 *Extraction*

The plant material (4 kg) was extracted successively with light petroleum distillate, ethyl acetate and methanol (6 × 15 litres of each). The extracts were evaporated under vacuum to give 31 g, 63 g and 330 g crude extracts, respectively.

2.2.3 *Isolation of petuniasterones* ***1–9*** *(Fig. 1)*

The crude petroleum extract was redissolved in light petroleum distillate (2 litres) and submitted to liquid–liquid separation with dioxane + water (1 + 1 by volume; 8 × 400 ml). The bioactive product from the dioxane + water layer (6 g) was applied to a silica gel column (400 × 45 mm) and eluted using a step gradient of different solvents and solvent mixtures with increasing polarity. The eluents were light petroleum + ethyl acetate (100 + 0; 98 + 2, 96 + 4,



	10		11
$R^1 = R^4 =$	2-methylbutanoyl-	$R^1 = R^4 =$	2-methylbutanoyl-
$R^2 =$	pentanoyl-	$R^2 =$	hexanoyl-
$R^3 =$	2-methylbutanoyl-	**11a**: $R^3 =$	2-methylbutanoyl-
		11b: $R^3 =$	3-methylbutanoyl-

	12		13
$R^1 = R^4 =$	2-methylbutanoyl-	$R^1 = R^4 =$	2-methylbutanoyl-
$R^2 =$	heptanoyl-	$R^2 =$	octanoyl-
12a: $R^3 =$	2-methylbutanoyl-	**13a**: $R^3 =$	2-methylbutanoyl-
12b: $R^3 =$	3-methylbutanoyl-	**13b**: $R^3 =$	3-methylbutanoyl-

Figure 1. Petuniasterones and sucrose esters isolated from *Petunia hybrida*.

92 + 8, 80 + 20, 70 + 30, 60 + 40, 50 + 50, 40 + 60, 30 + 70, 20 + 80, 10 + 90 by volume), ethyl acetate + methanol (100 + 0; 90 + 10, 80 + 20, 50 + 50 by volume) and methanol, using 500 ml solvent or solvent mixture in each step. Monitoring was done by TLC (solvent: light petroleum + ethyl acetate, 3 + 7 by volume) to give 20 fractions. Fractions 13 and 14 showed the highest biological activities.

Further separation of fraction 13 (595 mg) by reversed-phase MPLC (solvent: methanol + water (5 + 5 by volume), flow: 15 ml min^{-1}, detection: UV = 254 nm) and semi-preparative reversed-phase HPLC (solvent: acetonitrile + water, 6 + 4 by volume, flow: 5 ml min^{-1}, detection: UV = 254 nm) led to the isolation of petuniasterones **1** (1 mg), **2** (2.5 mg), **3** (35 mg), **4** (4 mg), **5** (14 mg), **6** (4 mg), **7** (2 mg), **8** (1 mg) and **9** (3 mg).

2.2.4 *Isolation of sucrose esters **10–13***

Final purification of fraction 14 (730 mg) on semi-preparative reversed-phase HPLC (LiChroCart 250-10; solvent: ethanol + water, 5 + 5 by volume, flow: 2.5 ml min^{-1}, detection: UV = 220 nm) yielded four groups of 2,3,4,6-tetra-*O*-acylated sucrose ester isomers with relative molecular masses of 678 (**10;** 2 mg), 692 (**11;** 28 mg), 706 (**12;** 64 mg) and 720 (**13;** 60 mg), respectively.

2.3 Biological activity

2.3.1 *Molluscicidal activity*

Bioassays were carried out with *Biomphalaria glabrata* Say (Planorbidae), one of the snail vectors of schistosomiasis. Snails of uniform shell sizes (8–10 mm diam) were used. Two snails were placed in a distilled water solution of the extracts, fractions or compounds containing ethanol (4 µl) and polyethylene glycol 400 (2 µl) ml^{-1} solution. Extracts and fractions were tested at 500, 200 and 100 µg ml^{-1}, and compounds were tested in a series of dilutions (100, 80, 60, 40, 20, 10 µg ml^{-1}) in order to establish the minimum concentrations required to kill both snails (100% mortality). Because very little material was available, the number of test replicates for each concentration was limited to two. Niclosamide (Bayluscide®; Bayer AG, Leverkusen, Germany) was used as a reference. It gave 100% mortality at 5 µg ml^{-1}.

After 24 hours, the snails were transferred to a vessel containing dechlorinated tap water, and mortality rates were determined after a 24-h recovery period.[1–3]

2.3.2 *Antibacterial activity by bioautography*

Test materials (200, 100 and 50 µg for extracts and fractions and 40, 20, 10, 5 and 1 µg for compounds) were applied to silica gel TLC sheets which were developed in an appropriate solvent system. The chromatograms were sprayed with suspensions of *Bacillus subtilis* (Ehrenberg) Cohn or *Pseudomonas stutzeri* (Lehmann & Neuman) Sijderius, and incubated in a humid atmosphere for 24 h at 30°C to permit growth of the bacteria. Subsequently, zones of inhibition were visualized by a tetrazolium salt: metabolically active bacteria convert the tetrazolium salt into the intensely coloured formazane and inhibition zones appear as white spots against a red background.[4]

3 RESULTS

3.1 Structure determination

Compounds **1–4, 6** and **8** were identified by comparison of their NMR spectra with published data.[5–7] The structures of the novel petuniasterones were determined by means of NMR experiments. Assignments were based upon [^{1}H], [^{1}H-^{1}H]COSY, [^{13}C], DEPT-, GHSQC- and HMBC-NMR spectra. The stereochemical arrangement in the side chain of petuniasterone **7** was proved by NOE experiments (22-H → 20-H, 23-H_b; 23-H_a → 21-H, 28-H; 28-H → 26-H, 27-H).

Chromatography of the sucrose ester mixture yielded four groups of compounds. The components in each group had identical masses, whereas the groups differed from each other by 14 mass units.

[^{1}H] and [^{13}C]NMR spectra of all groups showed the signals of sucrose molecules with the glucose moiety fully esterified, while the fructose portion showed four free hydroxy groups (Fig 1).[8]

[^{1}H-^{1}H]COSY spectra revealed 2-methylbutanoyl groups as major acyl residues for the sucrose ester isomers **10–13.** According to the HMBC spectra, the 2-methylbutanoyl groups were located at 2-O, 4-O and 6-O on the glucosyl moieties of all sucrose ester isomers. 2D NMR experiments indicated for **11–13** the presence of 3-methylbutanoyl groups as further possible acyl residues at position 4-O. The NMR spectra also suggested the presence of different straight-chain acyl groups linked to 3-O. The different molecular masses of the sucrose esters suggested that these straight-chain acyl groups were of different lengths.

The [^{1}H-^{1}H]COSY spectra, however, proved to be unsuitable for distinguishing the different straight-chain acyl groups because of the overlapping proton signals. Therefore, the chain lengths of the acyl groups attached to 3-O were determined by GHSQC-TOCSY. Thus, the signals arising from a pentanoyl (**10**), hexanoyl (**11**), heptanoyl (**12**) and octanoyl group (**13**) were observed.

3.2 Biological activity

All compounds were tested against the freshwater snail *B glabrata*, one of the intermediate hosts of schistosomiasis. We found that only those petuniasterones which contained either the bicyclic *ortho*ester as in **1** and **6** or bearing a 24,25-epoxy group and an esterified hydroxy group at position 22 as in **3–5** showed molluscicidal activity. All other

Table 1. Molluscicidal activities of petuniasterones and sucrose esters from *Petunia hybrida*

	Mortality at μg ml^{-1} (%)											
Compound[a]	*100*	*90*	*80*	*75*	*70*	*60*	*40*	*20*	*15*	*10*	*5*	*1*
1	100	–[b]	100	–	–	100	0	0	–	–	–	–
2	0	–	0	–	–	0	0	0	–	–	–	–
3	100	–	100	100	33	33	0	0	–	–	–	–
4	100	–	100	–	100	50	0	0	–	–	–	–
5	100	100	0	–	–	0	0	0	–	–	–	–
6	100	–	100	–	–	100	100	0	–	–	–	–
7	0	–	0	–	–	0	0	0	–	–	–	–
8	0	–	0	–	–	0	0	0	–	–	–	–
9	0	–	0	–	–	0	0	0	–	–	–	–
10	–	–	–	–	–	–	100	100	100	0	0	0
11	–	–	100	–	–	100	100	100	100	0	0	0
12	–	–	100	–	–	100	100	100	0	0	0	0
13	–	–	100	–	–	100	100	100	100	0	0	0

[a] **1** = petuniasterone A; **2** = petuniasterone C; **3** = petuniasterone C-22-*O*-acetate; **4** = petuniasterone C-22-*O*-[(methylthio)carbonyl]-acetate; **5** = petuniasterone C-22-*O*-[(methoxy)carbonyl]-acetate; **6** = petuniasterone D; **7** = (22*R*,24*R*)-22,24-epoxy-7,25-dihydroxyergosta-1,4-dien-3-one; **8** = petuniasterone G; **9** = petuniasterone G-22-*O*-acetate; **10** = sucrose ester isomers MW 678; **11** = sucrose ester isomers MW 692; **12** = sucrose ester isomers MW 706; **13** = sucrose ester isomers MW 720.
[b] – = not tested

petuniasterones were inactive against the snails at a concentration of 100 μg ml^{-1}. The sucrose esters **10**–**13** showed molluscicidal activities at concentrations as low as 15 μg ml^{-1}. Molluscicidal activities of the compounds are listed in Table 1.

Furthermore, the sucrose esters **10**–**13** were active against *B. subtilis* and *P. stutzeri* in the direct bioautography TLC assay at 5 μg per spot.

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